Interaction sites of the C-terminal region of the cGMP phosphodiesterase inhibitory subunit with the GDP-bound transducin α -subunit

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In the present report, the region of interaction between the GDP-bound α -subunit of transducin (α t.GTP) and the cGMP phosphodiesterase inhibitory γ -subunit ($P\gamma$) has been studied. It is widely accepted that the α t.GTP is the active form of transducin and that the GDP-bound transducin α -subunit (α t.GDP) is the inactive form. We have reported previously that the binding region of the C-terminal of $P\gamma$ on α t.GTP is in a region between the exposed face of the α 3 and α 4 helices of α t.GTP [Liu, Arshavsky and Ruoho (1996) *J. Biol. Chem.* **271**, 26900–26907]. We now report that *N*-[3-¹²⁵I]iodo-4-azidophenylpropionamido-S-(2-thiopyridyl)cysteine ([¹²⁵I]ACTP)-derivatized $P\gamma$ (at Cys-68) reversibly undergoes a unique disulphide exchange of the radioiodinated moiety *N*-(3-¹²⁵I)iodo-4-azidophenylpropionamido)cysteine ([¹²⁵I]APC) from Cys-68 of $P\gamma$ to α t.GDP but not to the guanosine 5'-(γ -thio)-triphosphate (GTP[S])-bound trans-

ducin α -subunit (α t-GTP[S]). The specificity of the interaction was demonstrated by the fact that exchange was protected by the functionally active Cys-68 $\rightarrow$ Ala $P\gamma$ mutant, and by pretreatment of the α t.GDP with the $\beta\gamma$ -subunit of transducin. Chemical cleavage and amino acid sequencing demonstrated that the [¹²⁵I]ACTP-derived $P\gamma$ specifically transferred the [¹²⁵I]APC group to Cys-250 and Cys-210 of α t.GDP. These data indicate that the C-terminal region (especially Cys-68–Trp-70) of $P\gamma$ interacts with α t.GDP on the exposed interface between α 2/ β 4 and α 3/ β 5 of the α -subunit of transducin. Disulphide exchange was also observed with the α -subunit of holotransducin but this was only approx. 60 % of that of pure α t.GDP. The variation in the binding pattern between α t.GDP and α t.GTP with the C-terminal region of $P\gamma$ may contribute to the functional difference between the GDP- and GTP-bound states.

INTRODUCTION

In vertebrate visual transduction systems, the heterotrimeric photoreceptor G-protein, transducin, mediates signal transfer between rhodopsin and intracellular enzymes and ion channels. Light-activated rhodopsin catalyses the exchange of GDP for GTP. After GTP binding of the α -subunit of transducin (α t.GTP), α t.GTP dissociates from the $\beta\gamma$ -subunit of transducin ($\beta\gamma$ T) and the disc membranes, binds to the inhibitory subunits ($P\gamma$) of phosphodiesterase (PDE) and releases the inhibitory action of $P\gamma$ on PDE α - and β -subunits ($P\alpha\beta$). The $P\alpha\beta$ becomes active and catalyses the hydrolysis of cGMP [1–8]. The signal is turned off by the hydrolysis of GTP, which is accelerated by the co-ordinated action of the photoreceptor-specific regulator of G-protein signalling RGS9 and $P\gamma$ [9–16] to convert $G\alpha$ from the GTP-bound form back to the GDP-bound form. Purified α t.GDP was found to bind to $P\gamma$ and it also activated PDE, but with a 100-fold lower affinity than that of α t.GTP [17–19]. The crystal structures of α t.GDP and α t.GTP presented by Lambright et al. [20] showed that there were three different regions of α t.GTP[S] and α t.GDP in the conformation which composed 14 % of the sequence; these were switch I, II and III. Although the binding pattern between α t.GTP and $P\gamma$ has not been determined finally, progress has been made in the understanding of the interaction domain(s) between α t.GTP and $P\gamma$. Current evidence indicates that there are two regions of $P\gamma$, the central region ($P\gamma$ 24–45) and the C-terminal region ($P\gamma$ 46–87), which bind to α t.GTP [10,21–25]. A 22 amino acid peptide, corresponding to residues 293–314 of the α -subunit of transducin, has been shown to mimic the α -subunit of transducin in activating PDE [26] and to bind to

the C-terminal of $P\gamma$ ($P\gamma$ 46–87) [27]. The central region of $P\gamma$ ($P\gamma$ 24–45) is thought to bind to residues 250–275 or to the α 4/ β 6 loop of the α -subunit of transducin [27–29]. Experiments reported by Spickofsky et al. [30] showed that five residues on one exposed face of the α 4 helix of rod transducin interacted with $P\gamma$. Previously, Liu et al. have identified the binding region for the C-terminal region of $P\gamma$ ($P\gamma$ 68–70) on α t.GTP[S], which is in a region between the exposed face of the α 3 and α 4 helices of the α -subunit of transducin [31]. Skiba et al. [32] also implied that the α 3 and α 4 helices of the α -subunit of transducin were directly involved in binding to $P\gamma$. However, less is known of the interaction between α t.GDP and its effector $P\gamma$, although α t.GDP has been shown to bind with lower affinity to $P\gamma$ and to activate PDE. In the present work, data is presented to show the region of interaction between the C-terminus of $P\gamma$ and α t.GDP using a unique method of radioisotope transfer via disulphide exchange.

EXPERIMENTAL

Chemicals

GTP, GTP[S], soybean trypsin inhibitor and N-ethylmaleimide (NEM) were from Sigma. Tosylphenylalanylchloromethane-treated trypsin and clostripain (sequencing grade) were purchased from Promega. The Mono S column and Blue-Sepharose CL-6B were obtained from Pharmacia–LKB Biotechnology. BNPS-skatole was obtained from Pierce; hydroxylamine hydrochloride was purchased from Fisher Scientific Company and cyanogen bromide (CNBr) was purchased from Aldrich Chemical Com-

Abbreviations used: PDE, phosphodiesterase; GTP[S], guanosine 5'-(γ -thio)-triphosphate; α t.GTP, GTP-bound transducin α -subunit; α t.GDP, GDP-bound transducin α -subunit; $\beta\gamma$ T, $\beta\gamma$ -subunit of transducin; α t-GTP[S], GTP[S]-bound transducin α -subunit; $P\gamma$, inhibitory γ -subunit of PDE; $P\alpha\beta$, α - and β -subunit of PDE; ACTP, *N*-[3-iodo-4-azidophenylpropionamido-S-(2-thiopyridyl)]cysteine; NEM, N-ethylmaleimide; [¹²⁵I]APC, *N*-(3-¹²⁵I)iodo-4-azidophenylpropionamido)cysteine; holo T, holotransducin; CNBr, cyanogen bromide.

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pany. The C4 reverse-phase HPLC column was obtained from Vydac Corporation.

Radiosynthesis of [125 I]ACTP

The radiosynthesis of 5 Ci/mmol [125 I]ACTP was accomplished by the reaction at room temperature for 12 h of carrier-free (2200 Ci/mmol) 3-[125 I]iodo-4-azidophenylpropionyl succinimide ([125 I]AIPPS) which had been appropriately adjusted with non-radioactive AIPPS and thiopyridyl cysteine [33]. [125 I]ACTP was purified by silica gel TLC, as described previously.

Preparation of α t.GTP[S] and α t.GDP

Holotransducin (holo T) was prepared from frozen dark-adapted bovine retinas (J. A. and W. L. Lawson Co., Lincoln, NE, U.S.A.) as described previously [34]. The holo T was then loaded on to a Blue-Sepharose CL-6B column, and the α t.GDP and β \gamma T were purified according to Yamazaki et al. [35]. The α t.GTP[S] was released from the bovine rod outer segment membranes using GTP[S] and purified on Blue-Sepharose CL-6B, as described by the method of Kroll et al. [36]. The purity of the α t- and β \gamma T-subunits was determined by SDS/PAGE. Staining with Coomassie Blue showed that the subunits were more than 95 % pure. The purified proteins were stored at -80°C and used within one month.

Preparation of P γ -[125 I]APC

The code sequence of P γ was subcloned into an expression vector pET-11a (Novagen). The vector was transformed into *Escherichia coli* strain BL21 DE3 strain [10,31,37] and grown in Luria Broth containing 100 $\mu\text{g}/\text{ml}$ of ampicillin until the D_{600} of the culture was 0.4–0.6, isopropyl β -D-thiogalactoside was then added to a final concentration of 5 mM. After incubation for 4 h at 30°C , the cells were harvested and stored at -80°C until use. Recombinant P γ was purified on a Mono S column and by reverse-phase HPLC (C4 column), as described previously [10,31,37]. Briefly, cells were suspended in 50 mM Tris/HCl, pH 7.6, containing protease inhibitors (PMSF, tosylphenylalanylchloromethane, leupeptin, aprotinin and pepstatin A), disrupted using a French press and centrifuged at 20000 r.p.m. ($\sim 50000\text{ g}$) for 20 min. The supernatant was loaded on to a Mono S column and eluted with a 0–1 M NaCl gradient in 50 mM Tris/HCl [$\sim 40\%$ (w/v) NaCl] using an FPLC system. The P γ -enriched fractions were loaded on to a C4 reverse-phase HPLC column and eluted with a 30–60 % (v/v) acetonitrile gradient containing 0.1 % (v/v) trifluoroacetic acid over a 40-min period (P γ eluted at approx. 40 % acetonitrile). The fractions containing P γ were pooled, freeze dried and stored at -80°C .

Derivatization of P γ was carried out by incubating P γ (100–200 μM) with 5 Ci/mmol [125 I]ACTP (1:2 molar ratio) in a solution (100–200 μl) containing 120 mM NaCl/10 mM Hepes/6 mM MgCl_2 , pH 7.6, at room temperature overnight [31]. The reaction mixture was passed through a C4 reverse-phase HPLC column and eluted with a 30–60 % (v/v) acetonitrile gradient containing 0.1 % (v/v) trifluoroacetic acid over 40 min with a flow rate of 0.5 ml/min. The [125 I]ACTP and underivatized P γ was eluted with approx. 35 % and 41 % (v/v) acetonitrile respectively, whereas P γ -[125 I]APC was eluted at approx. 45 % (v/v) acetonitrile. The purity of the eluted fractions was determined by SDS/PAGE (15 % gels) and autoradiography of the wet gel. P γ -[125 I]APC was estimated to be greater than 99 % pure. The P γ -[125 I]APC fractions were collected, freeze dried, stored at -20°C and used within one week.

Interaction of α t.GTP[S], α t.GDP and holo T with P γ -[125 I]APC

Purified α t.GDP, α t.GTP[S] or holo T (5–10 μM) were mixed with P γ -[125 I]APC (1:1 molar ratio) in 120 mM NaCl/6 mM MgCl_2 /10 mM Hepes, pH 7.6, and incubated at room temperature for 30 min. The mixtures were photolysed for 6 s in ice-water at a distance of 10 cm from a water-jacketed 1 kW high-pressure Hg lamp [31,32] or were kept in the dark. Free cysteine residues were then modified using 4 mM NEM and protein was denatured by the addition of 2 % (w/v) SDS. The samples were subjected to SDS/PAGE (12 % gels).

Chemical cleavage of α t.GDP-[125 I]APC disulphide-exchanged product

The radioligand-transferred α t.GDP (α t.GDP-[125 I]APC) band was excised from the polyacrylamide gel and eluted with water overnight. The eluted the α -subunit of transducin was desalted on a 3-ml Sephadex G50 column.

BNPS-skatole cleavage

The radioligand-transferred α t.GDP was dissolved in 10 μl of water, 30 μl of BNPS-skatole/acetic acid (1.3 mg/ml) was added and the solution was incubated at 47°C for 2 h [31,38,39].

CNBr cleavage

CNBr cleavage was performed in 70 % (v/v) formic acid/0.2 M CNBr in the absence of O_2 for 24 h [31,40]. The sample was then freeze dried and the products were separated by tricine/PAGE (16.5 % polyacrylamide gel).

Hydroxylamine cleavage

α t.GDP was incubated with 2 M hydroxylamine/0.2 M K_2CO_3 , pH 10.5, at 42°C for 3 h and the products were separated by SDS/PAGE (12 % gel) [41,42]. After electrophoresis, the proteins or peptides were transferred to PVDF (Bio-Rad, 0.2 μm) in 10 mM 3-(cyclohexylamino)propane-1-sulphonic acid/20 % (v/v) methanol, pH 11, at 5 mA for 60 min using a Hoefer trans-blot apparatus.

Analytical methods

Automatic N-terminal protein sequencing was performed using an ABI, Model 470A, gas-phase sequencer equipped with an on-line Model 120A phenylthiohydantoin analyser. Microsequencing and radiosequencing were performed at the Supramolecular Structure Facility (Michigan State University, East Lansing, MI, U.S.A.). SDS/PAGE was performed by the method of Laemmli [43]. Tricine/PAGE was carried out as described by Schagger and von Jagow [44]. Protein concentrations were determined by the Coomassie Blue binding method [45] using BSA as a standard or spectrophotometrically at $A_{280\text{ nm}}$ using a molar absorption coefficient of $7100\text{ M}^{-1}\cdot\text{cm}^{-1}$ for P γ [10]. Densitometric scans of the Coomassie-Blue-stained gels and autoradiographic film were performed on a Model SL-504XL Zeineh Soft Laser scanning densitometer. Computer modelling was performed in the laboratory of Dr. Robert Fillingame (Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI, U.S.A.) with Unix Silicon Graphics using the Insight II (Version 2.3.0) Biosym Technologies program.

Figure 1 Specific disulphide exchange between P γ -[¹²⁵I]APC and α t.GDP

(Upper panel) P γ (Cys-68 $\rightarrow$ Ala) and β γ T protection against specific disulphide exchange between P γ -[¹²⁵I]APC and α t.GDP: (a) Coomassie Blue staining and (b) autoradiogram of an SDS/polyacrylamide gel (15%). α t.GTP[S] (α t.GTP γ S), α t.GDP or holo T at a concentration of 5–10 μ M, were preincubated with or without a 4-fold molar excess of C68A P γ and/or β γ T [over the α -subunit of transducin (α t)] for 5 min, followed by a stoichiometric addition of P γ -[¹²⁵I]APC. After incubation for 30 min at room temperature, samples were kept in the dark or photolysed; 4 mM NEM and 2% SDS was then added and the samples were analysed by PAGE (15% gel) (the molecular masses were: α t, 37 kDa; β t, 35 kDa; α t-P γ , 47 kDa). (Middle panel) 2-Mercaptoethanol treatment of disulphide-exchanged α -subunit of transducin removes the radiolabel from the α -subunit of transducin (α t). α t.GDP was mixed with P γ -[¹²⁵I]APC (1:1 ratio) at a concentration of 5–10 μ M and incubated for 30 min at room temperature, after which samples were kept in the dark or photolysed. NEM (4 mM) and 2% (w/v) SDS was added and the samples were treated with (lanes 3 and 4) or without (lanes 1 and 2) 2% (v/v)

Figure 2 Time course of disulphide exchange between P γ -[¹²⁵I]APC and the α -subunit of transducin

α t.GDP (5 μ M) ($\square$) or α t.GTP[S] (5 μ M) ($\bullet$) was incubated with P γ -ACTP (1:1 molar ratio) at room temperature in the dark. At the times shown, the reactions were terminated by adding 4 mM NEM followed by 2% (w/v) SDS. The samples were then analysed as described in the legend to Figure 1. The intensity of the autoradiograph bands was laser scanned and the data used to plot the curve. The data are representative of two experiments.

RESULTS

Characterization of specific interaction of P γ -[¹²⁵I]APC with α t.GDP and the α -subunit of holo T

α t.GTP[S] binds tightly to P γ in solution. We have shown previously that the derivatization of P γ with photo-reagent, ACTP, does not influence the interaction between α t.GTP and P γ [31]; after photolysis, α t.GTP[S] forms a cross-link with P γ -[¹²⁵I]APC. The α t.GDP binds to P γ with only low affinity [19,35,46]. When α t.GDP and P γ -[¹²⁵I]APC were incubated together for 30 min, a cross-linkage with an approx. 50% yield, measured by a laser scanning of the autoradiogram, formed after photolysis (Figure 1, upper panel, part b) compared with that of α t.GTP[S] (Figure 1, upper panel, part b; lanes 2 and 4). Interestingly we found that in the dark condition, α t.GDP became radioactive (Figure 1, upper panel, part b; lane 3). This radioactivity could be removed by treating it with 2-mercaptoethanol (Figure 1, bottom panel, lane 3), indicating that the connection between the α t.GDP and the radioactivity was via a disulphide bond. One explanation is that, whereas P γ -[¹²⁵I]APC is bound to α t.GDP, Cys-68 of P γ is positioned so close to the cysteine(s) of α t.GDP that the [¹²⁵I]APC group in Cys-68 of P γ is transferred to the cysteine(s) of α t.GDP via disulphide exchange. The possibility of contamination of P γ -[¹²⁵I]APC with free [¹²⁵I]ACTP was excluded by: (1) separation of P γ -[¹²⁵I]APC from free [¹²⁵I]ACTP by HPLC and monitoring of the P γ -[¹²⁵I]APC fractions by SDS/PAGE; almost 99% of the radioactivity was in the P γ position [31], and (2) free [¹²⁵I]ACTP readily derivatized α t.GTP[S], α t.GDP and β γ T (results not shown), but in the control (Figure 1, upper panel, part b; lane 1), α t.GTP[S] had little radioactivity and there was no radioactivity seen in β γ T (Figure 1, upper panel, part b; lanes 5–8 and 11–14). Most

2-mercaptoethanol and analysed by PAGE (15% gel). (Lower panel) α t.GTP[S] protection on α t.GDP and P γ -[¹²⁵I]APC disulphide exchange. α t.GTP[S] and α t.GDP were mixed with P γ -APC under the conditions described in the Experimental section. The results are representative of three to four experiments.

Scheme 1 Disulphide exchange and the cleavage schemes for the α -subunit of transducin

[125 I]ACTP derivatized P γ at Cys-68, forming P γ -[125 I]APC and thiopyridone. P γ -[125 I]APC was further purified free of P γ and [125 I]ACTP using HPLC. After mixing P γ -[125 I]APC and α t.GDP (1:1 molar ratio) in the dark, the [125 I]APC group in P γ -[125 I]APC was transferred to α t.GDP forming α t.GDP-[125 I]APC. Since the α -subunit of transducin contains two tryptophan residues at positions 127 and 207, the cleavage of the α -subunit of transducin with BNPS-skatole yielded three complete peptides with apparent molecular masses of 14 kDa (residues 1–127), 9 kDa (residues 128–207) and 15 kDa (residues 208–349). Only the 15 kDa peptide, sequence 208 to the C-terminus, was [125 I]-labelled (see Figure 3). Radiosequencing of the 15 kDa fragment showed that the [125 I]-label was released at the third cycle, and not at the ninth cycle, which correspond to Cys-210 and Cys-216 respectively. The α -subunit of transducin was further cleaved with CNBr; a 12 kDa fragment was produced, which was radioactive. Microsequencing of the 12 kDa peptide indicated that it was composed of two fragments. The N-terminus of the major fragment began at His-240 and probably extended to the end of the C-terminus of the α -subunit of transducin; another minor fragment started from Ser-119. Radiosequencing indicated that the [125 I]-label was released at the eleventh cycle, corresponding to the Cys-250 of the major peptide and Ser-129 of the minor peptide (see Figure 5). Since no radioactivity was found on the α t.GDP residues 1–207 (Figure 3) and serine was not able to form a disulphide bond with the cysteine residue, it was concluded that the radioactivity released was from the Cys-250 of the α -subunit of transducin. Since the α -subunit of transducin contains one Asn-287–Gly-288 bond, cleavage of the subunit with hydroxylamine yielded two major fragments with molecular masses of approx. 33 kDa and 6 kDa. The 33 kDa fragment contained most of the radioactivity (95%) (see Figure 4). It was concluded that Cys-210 and Cys-250 of the α -subunit of transducin are specifically disulphide exchanged with P γ -[125 I]APC. The solid lines represent the peptides which were [125 I]-labelled, while the broken lines indicates unlabelled fragments.

importantly, this radioactive exchange was specifically protected by excess Cys-68 $\rightarrow$ Ala (C68A) P γ as well as by $\beta\gamma$ T. A 4-fold excess of $\beta\gamma$ T partially protected α t.GDP from the disulphide exchange ($\sim 50\%$) (Figure 1, upper panel, part b; lane 5); a 4-fold excess of C68A P γ largely protected the α t.GDP from disulphide exchange (~ 80 – 90% protection) (Figure 1, upper panel, part b, lane 9). A combination of $\beta\gamma$ T and C68AP γ almost completely blocked the disulphide exchange (Figure 1, upper panel, part b, lane 7). The α -subunit of holo T also showed disulphide exchange, although the extent was about 60–70% of that of pure α t.GDP (Figure 1, upper panel, part b, lane 11). When α t.GTP[S] and α t.GDP were pretreated with NEM, which derivatizes the exposed sulphhydryl groups [47,48], the disulphide exchange between α t.GDP and P γ -[125 I]APC was blocked (results not shown). Under these conditions, α t.GDP was still able to

form the cross-linkage with P γ -[125 I]APC but with a lower yield (approx. 20% of that of α t.GTP[S]). This might be because pretreatment with NEM abolished the photo-crosslink from α t.GDP-[125 I]APC to P γ . Preincubation of α t.GTP[S] with P γ in a 2:1 molar ratio at 0–4 °C or at 25 °C for 2 min almost completely inhibited the disulphide exchange between α t.GDP and P γ (1:1 molar ratio) (Figure 1, middle panel, lanes 4 and 6), and the preincubation of α t.GTP[S] with P γ in a 1:1 molar ratio at 0–4 °C or 25 °C for 2 min (-2 min, Figure 1, middle panel) inhibited the disulphide exchange between α t.GDP and P γ by more than 90% (Figure 1, middle panel, lanes 3 and 5). Even if α t.GDP and P γ -[125 I]APC were preincubated at 25 °C for 2 min and then a 2:1 or 1:1 molar ratio of α t.GTP[S] was added ($+2$ min; Figure 1, middle panel), the disulphide exchange between α t.GDP and P γ was blocked by $> 90\%$ (Figure 1,

Figure 3 Identification of the disulphide exchange site of $P\gamma$ - $[^{125}\text{I}]\text{APC}$ on the 15 kDa BNPS-skatole fragment of $\alpha\text{t.GDP}$

After disulphide exchange of $\alpha\text{t.GDP}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$, $[^{125}\text{I}]\alpha\text{t.GDP}$ was separated by PAGE (12% gel), eluted with water and cleaved with BNPS-skatole. (a) Coomassie Blue staining and autoradiogram of PVDF-transferred 15 kDa BNPS-skatole fragment. (b) Radiochemical sequencing of the 15 kDa polypeptide BNPS-skatole fragment indicated that the ^{125}I -label was released on the third cycle, which corresponds to Cys-210; no radioactivity was released on the ninth cycle, which corresponds to Cys-216. These results are representative of two to three experiments.

middle panel, lanes 7 and 8). This is consistent with the observations that $\alpha\text{t.GTP[S]}$ has much higher affinity for $P\gamma$ than $\alpha\text{t.GDP}$, therefore $\alpha\text{t.GTP[S]}$ was able to compete with $\alpha\text{t.GDP}$ for the binding of $P\gamma$.

A time course of the disulphide exchange showed that the exchange between $\alpha\text{t.GDP}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$ reached an equilibrium in about 20 min but the exchange was much slower between $\alpha\text{t.GTP[S]}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$. There was less than 10% radioactivity in $\alpha\text{t.GTP[S]}$ after incubation with $P\gamma$ - $[^{125}\text{I}]\text{APC}$ at room temperature for 120 min (Figure 2).

Identification of the cysteine residues in $\alpha\text{t.GDP}$ specifically targeted by $P\gamma$ - $[^{125}\text{I}]\text{APC}$

Cleavage of the α -subunit of transducin with BNPS-skatole and hydroxylamine

To identify the disulphide exchanged cysteine(s), the $\alpha\text{t.GDP}$, which had been eluted from a polyacrylamide gel, was treated with BNPS-skatole. Three completely cleaved fragments (14, 15 and 9 kDa) and two incompletely cleaved fragments (24 and 25 kDa) were obtained (Scheme 1), as predicted from the results of previous work [31,49]. We found that, of the completely cleaved fragments, only the 15 kDa peptide (residues 208–349 of the α -subunit of transducin) was radioactive (Figure 3a); neither the 14 kDa (residues 1–127) nor the 9 kDa (residues 128–207) peptide contained radioactivity. This indicated that the disulphide-exchanged cysteines were in the C-terminal 15 kDa peptide (residues 208–350) and not in the N-terminal residues (1–207). Thus Cys-62 and Cys-135 were excluded as candidates for disulphide exchange, since the 14 kDa and 9 kDa fragments were not radiolabelled. There were a total of six cysteine residues in the 15 kDa peptide. Radiosequencing of the BNPS-skatole 15 kDa peptide showed that most of the radioactivity was released at the third cycle, which corresponded to Cys-210. No release of radioactivity was found at the ninth cycle (Figure 3b), indicating that Cys-216 was unlikely to be involved in the disulphide exchange and this residue was excluded from further consideration.

Figure 4 Hydroxylamine cleavage of $\alpha\text{t.GDP}$

After disulphide exchange of $\alpha\text{t.GDP}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$, $[^{125}\text{I}]\alpha\text{t.GDP}$ was separated by PAGE (12% gel), eluted with water and cleaved with hydroxylamine, as described in the Experimental section. A 33 kDa fragment [residues 1–287 of the α -subunit of transducin (αt)] and a 6 kDa fragment (residues 288–349 of the α -subunit of transducin) were produced. The majority of the radioactivity (more than 95%) was found in the 33 kDa fragment and less than 5% of the radioactivity remained in the 6 kDa fragment.

When the derivatized $\alpha\text{t.GDP}$ was cleaved with hydroxylamine, which produced a 33 kDa fragment (residues 1–287 of the α -subunit of transducin) and a 6 kDa fragment (residues 288–349) (Scheme 1), the majority of the radioactivity was found in the 33 kDa fragment, and less than 5% was in the 6 kDa fragment (Figure 4). This excluded Cys-321 and Cys-347 (which are near the C-terminus of the α -subunit of transducin) for disulphide exchange.

Figure 5 Analysis of $\alpha\text{t.GDP}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$ disulphide exchange by CNBr cleavage

After disulphide exchange of $\alpha\text{t.GDP}$ and $P\gamma$ - $[^{125}\text{I}]\text{APC}$, $[^{125}\text{I}]\alpha\text{t.GDP}$ was separated by PAGE (12% gel), eluted with water and cleaved with CNBr for 24 h. The sample was then separated on a tricine gel (16.5% polyacrylamide, 3% crosslinker) and transferred to a PVDF membrane. Autoradiography of the gel showed a 12 kDa fragment (a), which, on chemical and radioactive sequencing (b), provided two sequences. The first ten residues represented the actual sequence obtained from N-terminal microsequencing. Radiosequencing indicated that ^{125}I -label was released on the eleventh cycle, which corresponded to Cys-250 and Ser-129. Since no radioactivity was observed in $\alpha\text{t.GDP}$ residues 1–207 (see Scheme 1 for summary of the disulphide exchange and cleavage of the α -subunit of transducin), and there is no cysteine residue within the first 15 residues of the peptide having an N-terminus of Ser-119, we concluded that the ^{125}I -label was released from Cys-250. The results shown are representative of two to three experiments.

Figure 6 Molecular modelling of the interaction between α t.GDP and the Cys-68–Trp-70 sequence of $P\gamma$

The location of $P\gamma$ Cys-68, based on the data given in the present study, was superimposed on to the crystal structure of α t.GTP[S] and α t.GDP (from [50]). The purple and green ribbons represent the α -subunit of transducin and $P\gamma$ respectively. Side chains are coloured as follows: red, His-244, Met-308 and Arg-310 of the α -subunit of transducin are photo-insertion sites; yellow, Cys-210 and Cys-250 of the α -subunit of transducin and Cys-68 of $P\gamma$; white, Trp-70 of $P\gamma$; and green, Pro-69 of $P\gamma$. White indicates the residues from the α -subunit of transducin, whereas yellow indicates the residues from $P\gamma$.

Cleavage of the α -subunit of transducin with CNBr

To analyse the Cys-250 derivatization of α t.GTP, we treated the α -subunit of transducin with CNBr and produced a 12 kDa fragment (Scheme 1), which was found to be radioactive (Figure 5a). N-terminal microsequencing revealed that it was composed of two fragments. The N-terminus of the major fragment began at residue 240 and extended, in all likelihood, to the end of the C-terminus of the α -subunit of transducin; the other minor fragment started at residue 119. Radiosequencing indicated that the ^{125}I -label was released at the eleventh cycle, corresponding to Cys-250 of the major peptide and Ser-129 of the minor peptide (Figure 5b). Since no radioactivity was found associated with residues 1–207 of α t.GDP (Figure 4), and serine could not form a disulphide bond with cysteine, we concluded that the radioactivity released was from Cys-250 of the α -subunit of transducin. Previously, it had been reported that Cys-347 of the α -subunit of transducin, derivatized with ACTP, could exchange [^{125}I]APC with the Cys-210 of the α -subunit of transducin [33], since these two cysteine residues are very close to each other in the structure [20,50]. It is possible that the low yield of radioactivity (5%) in the small hydroxylamine fragment was caused by the transfer of [^{125}I]APC from Cys-210 to Cys-347 of α t.GDP. Also, Cys-282 of the α -subunit of transducin is on the opposite side of the peptide from Cys-210 and Cys-250 (according to the crystal structure [50]). We therefore concluded that $P\gamma$ -[^{125}I]APC disulphide exchange occurs specifically with Cys-210 and Cys-250 of α t.GDP.

In summary, we conclude that the regions of interaction between the $P\gamma$ C-terminal region of α t.GTP and α t.GDP are different. The binding region between the α t.GTP form and the C-terminal region of $P\gamma$ ($P\gamma$ 68–70) is between the exposed face of the α 3 and α 4 helices of the α -subunit of transducin, as shown previously [31] and in Figure 6 (right). In the GDP form, however, the α -subunit of transducin binds the $P\gamma$ C-terminal region less tightly. The binding region of α t.GDP and the C-terminal region of $P\gamma$ ($P\gamma$ 68–70) occur between Cys-210 and Cys-250 of α t, as illustrated by the computer model (Figure 6, left).

DISCUSSION

As shown in Figure 1, $P\gamma$ -[^{125}I]APC specifically transferred radioactivity to α t.GDP but not to α t.GTP[S] under dark conditions. One interpretation of the data is that Cys-68 of $P\gamma$, which is derivatized by ACTP via a disulphide bond, is in very close proximity to the cysteine(s) on the α -subunit of transducin, so that $P\gamma$ -[^{125}I]APC disulphide exchanges its APC moiety to α t.GDP. The cysteine disulphides which are exchanged must be in or near the region of interaction between α t.GDP and $P\gamma$ for the exchange to be specific. The fact that a 4-fold excess of C68A $P\gamma$ blocked 80–90% of the disulphide exchange between $P\gamma$ -[^{125}I]APC and α t.GDP indicated that there was direct interaction between the C-terminal region of $P\gamma$ and α t.GDP.

Of the eight cysteines in α t.GDP, the two cysteines in residues 1–207 were excluded as reactants, based on the BNPS-skatole

cleavage results (Figure 3). Cysteine residues 347 and 321 contained less than 5% of the radioactive label and were thus also unlikely to take part in the disulphide exchange. Three of the four remaining cysteines (Cys-210, Cys-216 and Cys-250) were further examined. Cys-210 and Cys-250 were found to be ^{125}I -labelled and therefore involved in the disulphide exchange but, on radiosequencing, no radioactivity was seen to be released from Cys-216, thus excluding this cysteine from the disulphide exchange. Cys-282 was not considered to be a target for disulphide exchange because it is on the opposite face of the α -subunit of transducin from Cys-210 and Cys-250.

Cys-210 and Cys-250 are juxtaposed, according to the crystal structure, and are thus located on the same face of the α -subunit of transducin [20,50]. These results indicate that the interaction domain of the C-terminal region of $\text{P}\gamma$ (especially $\text{P}\gamma$ 68–70) is in a region between the $\alpha 2$ – $\alpha 2/\beta 4$ loop and the $\alpha 3$ – $\alpha 3/\beta 5$ loop of $\alpha\text{t.GDP}$. It is possible that the interaction between $\alpha\text{t.GDP}$ and the C-terminal region of $\text{P}\gamma$ may not be of high affinity, so that Cys-68 of $\text{P}\gamma$ is sufficiently flexible while bound to the surface of the α -subunit of transducin to derivatize either Cys-210 or Cys-250.

$\alpha\text{t.GDP}$ specifically formed a cross-linkage with $\text{P}\gamma$ - ^{125}I APC when photolysed (Figure 1, upper panel, lanes 7 and 9) with a 20–50% yield compared with that of $\alpha\text{t.GTP[S]}$, which is consistent with the report from Hamm and colleagues [28]. Previously, we had identified three residues in the $\alpha\text{t.GTP[S]}$ form which were photolabelled by $\text{P}\gamma$ -APC; namely, His-244, Met-308 and Arg-310. The photoinsertion domain on $\alpha\text{t.GDP}$ from $\text{P}\gamma$ - ^{125}I APC was not investigated further for the following technical reasons: the photoinsertion was not as efficient as that for the $\alpha\text{t.GTP[S]}$ interaction (20–50%) (Figure 1) and much lower than the disulphide exchange (less than 10%). The specificity of the disulphide exchange from $\text{P}\gamma$ - ^{125}I APC to $\alpha\text{t.GDP}$ was demonstrated by several observations: (1) 80–90% protection was afforded by a 4-fold excess of C68A $\text{P}\gamma$ (Figure 1, upper panel, lane 9), (2) approx. 50% protection by 4-fold $\beta\gamma\text{T}$ (Figure 1, upper panel, lane 5) and (3) almost complete protection by $\alpha\text{t.GTP[S]}$ (Figure 1, middle panel).

$\text{P}\gamma$ has been observed to bind to $\alpha\text{t.GDP}$ and the $\alpha\text{t.GDP-P}\gamma$ complex can be removed from the disc membrane as well as dissociate from the catalytic core of PDE ($\text{P}\alpha\beta$) under certain conditions [18,35,46]. In addition, $\alpha\text{t.GDP}$ is able to fully activate PDE, albeit with a lower affinity than that of $\alpha\text{t.GTP}$ [19]. Direct evidence concerning the interaction among the α -, β - and γ -subunits of G-proteins has been presented from the crystal structure of G-protein heterotrimers by two groups [51,52]. It is clear that the N-terminus and switches I and II of the $\text{G}\alpha$ -subunit are involved in the $\text{G}\beta\gamma$ -subunit contact. The Cys-210 of the α -subunit makes van der Waals contact with Tyr-59 in $\text{G}\beta\gamma$. The data from the study presented in this paper show that $\beta\gamma\text{T}$ partially (approx. 50%) blocked the disulphide exchange between $\alpha\text{t.GDP}$ and $\text{P}\gamma$ - ^{125}I APC. As shown in Figure 1 (upper panel, lane 11), the α -subunit of holo T was also disulphide exchanged, but the level of radioactivity was approx. 50–60% of that of the pure $\alpha\text{t.GDP}$, by laser scanning of the autoradiogram. To establish whether the reduction in radioactivity in holo T was due to Cys-250 or Cys-210, the radioiodine released from Cys-210 was quantified. The amount of derivatization of Cys-210 of the α -subunit of holo T was approx. 20% of that observed for the α -subunit of transducin from the Cys-210 of the pure $\alpha\text{t.GDP}$, based on results using the same amount of protein in a parallel experiment (results not shown). This indicated that $\beta\gamma\text{T}$ specifically blocked the disulphide exchange from $\text{P}\gamma$ - ^{125}I APC to the Cys-210 of $\alpha\text{t.GDP}$, most likely due to the binding of $\beta\gamma\text{T}$ to the $\alpha\text{t.GDP}$ in the $\alpha 2$ helix region, which blocks the accessibility of

Cys-210 to ^{125}I APC in $\text{P}\gamma$. These results indicate that the $\alpha 2$ and/or $\alpha 2/\beta 4$ loop is the binding region between the α -subunit and the $\beta\gamma$ -subunit of transducin, which is totally consistent with the crystal structure of the heterotrimeric G-proteins [51,52]. The difference in interaction between $\alpha\text{t.GDP}$ and $\alpha\text{t.GTP}$ with $\text{P}\gamma$ may be due to the fact that $\alpha\text{t.GTP}$ binds the Cys-68 region of $\text{P}\gamma$ very tightly in the $\alpha 3$ and $\alpha 4$ helical domains of the transducin α -subunit. In $\alpha\text{t.GDP}$, the binding between the transducin α -subunit and the $\text{P}\gamma$ C-terminal region (i.e. Cys-68) is more flexible and allows the Cys-68 of $\text{P}\gamma$ to engage in disulphide exchange with Cys-210 and Cys-250 of the transducin α -subunit. This is supported by the disulphide exchange time courses (Figure 2), in which the disulphide exchange was observed between $\alpha\text{t.GTP[S]}$ and $\text{P}\gamma$ - ^{125}I APC, albeit at a much slower rate than that between $\alpha\text{t.GDP}$ and $\text{P}\gamma$ - ^{125}I APC.

It has been reported that hydrolysis of the bound GTP by the α -subunit of transducin was necessary for the termination of PDE activity but it was unclear whether it is sufficient for PDE shut-off [12,53]. $\beta\gamma\text{T}$ was required to dissociate the $\alpha\text{t.GDP-P}\gamma$ complex [18,35,46] and it has been hypothesized that $\beta\gamma\text{T}$ is involved in the true shut-off step [19,35]. The affinity between $\alpha\text{t.GTP}$ and $\text{P}\gamma$ is at least 100-fold higher than that of $\alpha\text{t.GDP}$ and $\text{P}\gamma$. The inhibition of trypsin-treated PDE activity by $\alpha\text{t.GDP}$ was not reversible by $\alpha\text{t.GTP[S]}$ but could be reversed by $\beta\gamma\text{T}$. This might indicate that $\alpha\text{t.GTP}$ directly interacts with $\text{P}\alpha\beta$ [36]. In agreement with this observation, formation of $\alpha\text{t.GTP[S]-P}\alpha$ and $\alpha\text{t.GTP[S]-P}\beta$ complexes was shown by Clerc et al. [54] in a cross-linking study of the proteins. The binding site for $\text{P}\alpha\beta$ is likely to be in the central region of $\text{P}\gamma$ ($\text{P}\gamma$ 24–45), whereas the C-terminal domain of $\text{P}\gamma$ is the region which confers inhibition of $\text{P}\alpha\beta$, and is believed to be involved in GTPase accelerating activity [10] and binding of the α -subunit of transducin [27,28]. The activation of PDE could involve the binding of $\alpha\text{t.GTP}$ to $\text{P}\gamma$, causing it to be physically removed from $\text{P}\alpha\beta$ or, perhaps more likely, when $\alpha\text{t.GTP}$ binds $\text{P}\gamma$, the complex with $\text{P}\alpha\beta$ remains in the membrane. Membrane-bound $\alpha\text{t.GTP}$ had been shown to be more efficient than soluble $\alpha\text{t.GTP}$ [55]. The physical state of the $\alpha\text{t.GDP}$ in the membrane may play an important role in the interaction with PDE. The finding that the α -subunit of transducin forms dimers and perhaps trimers [49,56,57] supports the hypothesis that G-proteins may function as oligomers [34,58,59]. The crystal structure of $\text{G}_i\alpha 1$ [60] shows that the amino terminus of the α -subunit of G_i forms extensive quaternary contacts with neighboring α -subunits in the lattice in a head to tail arrangement, strongly supporting the multimers hypothesis of α -subunits or heterotrimers. The precise interaction of such $\alpha\text{t.GDP}$ oligomers with subunits of the membrane-bound PDE has yet to be determined, but may involve contacts between the $\alpha\beta$ -subunit and the γ -subunit of PDE.

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